Acetylcholine, carbachol, and GABA induce no detectable change in the length of isolated outer hair cells

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The mechanical and electrical properties of cochlear outer hair cells (OHCs) are suggested to modulate transduction by inner hair cells. These properties of OHCs are presumably regulated by efferent neurons which use several transmitters including acetylcholine (Ach) and gamma aminobutyric acid (GABA). Since it had been suggested that Ach causes isolated OHCs to shorten visibly, this study was designed to investigate whether GABA also alters the length of OHCs. OHCs were isolated from the guinea pig cochlea by mechanical dispersion after collagenase treatment. Cells were initially selected by strict morphological criteria. In addition they were only included in further studies if they attained a constant length during 10 min of superfusion with buffer solution. Neither GABA (20 μM; 100 μM), Ach (5 mM; 10 μM with 10 μM eserine) or carbachol (10 μM; 100 μM) altered OHC length when applied in iso-osmotic Hank's balanced salt solution (total number of cells tested, 72). If a change in length occurred it must have been smaller than 0.3 μm, our detection ability. In contrast, high potassium and variations in osmolarity changed hair cell length by 3–10% in agreement with other reports.

Hair cells, outer; Gamma aminobutyric acid; Acetylcholine; Shorten; Motility

Introduction

Isolated OHCs have recently been studied in vitro where they have been shown to change length with slow time constants in the msec range in response to chemical and physical stimuli (Goldstein and Mizukoshi, 1967; Zener et al., 1985a,b; Brownell 1984; Brownell et al., 1985; Zener, 1986a,b; 1988; Schacht and Zener, 1987; Dulon et al., 1987, 1988) and with faster time constants in the μs range in response to electrical stimuli (Brownell, 1983, 1984; Brownell et al., 1985; Kachar et al., 1986; Ashmore, 1987). This property of the OHCs has been used to explain their modulatory role in transduction and the extreme sensitivity to sound energy of the mammalian cochlea. Now most models of cochlear function propose a role for the OHCs and their motility in a cochlear amplifier model (Davis, 1983), a negative damping model (Mountain, 1986) or other models (Dallos, 1985; Kim, 1986; Allen, 1988).

Efferent nerve fibers of the olivocochlear bundle (OCB) synapse on the OHCs. The mechanism whereby these efferent nerve fibers modulate the afferent output of the cochlea is unknown. Wiederhold (1986) states in a recent review of the role of the efferents, 'Thus,... the most parsimonious explanation of efferent suppression of neural responses is that medial OCB activation changes the mechanical properties of the OHCs and/or their stereocilia, which changes the mechanical coupling of basilar membrane displacement to IHC [inner hair cell] stereocilia deflection.'
Since 1978 investigators have speculated that efferent neurons innervating the OHCs change the length of the OHCs or the tension their stereocilia exert on the tectorial membrane (as discussed in: Bobbin and Kisiel, 1981) but it remains unknown whether the efferents actually change the length of OHCs in vivo. Brownell (1983, 1984; Brownell et al., 1985) was the first to suggest that iontophoretically applied Ach, the neurotransmitter of the majority of the efferents, evoked a shortening of isolated OHCs. Slepecky et al. (1988b) report that they observed a shortening of OHCs greater than 1 μm followed by elongation utilizing 0.5 and 5 mM acetylcholine applied as a solution to drops containing isolated OHCs. In addition Plinkert and Zemper (1989) in their oral presentation report that carbachol, a stable acetylcholine-like agonist, shortens isolated OHCs in about 60% of the cells observed.

While Ach is the transmitter at a majority of the efferent neurons innervating the cochlea, a subpopulation of efferents may utilize GABA as a transmitter (Altschuler and Fex, 1986). Therefore one of the purposes of this study was to test the hypothesis that GABA induces a change in the length of isolated OHCs in a similar fashion to that reported for Ach. In addition, we attempted to duplicate the effects on OHC shape of several agents studied previously by others including: hyper- and hypo-osmotic solutions, high potassium solutions, Ach, and carbachol.

Methods

Guinea pigs were anesthetized with pentobarbital (Nembutal, 30 mg/kg, i.p.), decapitated, and outer hair cells isolated from the cochlea essentially as described by others (Zenner et al., 1985a, 1985b, 1988; Zajic and Schacht, 1987; Dulon et al., 1988). The bulla of both ears was removed from the animal, opened and placed in Hank's balanced salt solution at room temperature containing (mM): NaCl, 137; KCl, 5; CaCl₂, 1.2; MgCl₂, 0.49; MgSO₄, 0.4; KH₂PO₄, 0.44; Na₂HPO₄, 0.33; D-glucose, 5.55; HEPES, 5; the pH was adjusted to 7.4 with 4 N NaOH; HBS, Gibco Labs). The osmotic pressure of the HBS was measured with a vapor pressure osmometer (Wescor). The unadjusted osmotic pressure was about 280 mOs and 4 M NaCl was added until the desired pressure was obtained (usually 304 mOs).

The modiolus including turns 3 and 4 of the organ of Corti from both ears was removed and transferred to a drop (100 μl) of HBS containing collagenase (Sigma Type IV; 1 mg/ml) for 10 min. Subsequent dissection was carried out in HBS by micromanipulation in the absence of proteolytic enzyme which had been removed by rinsing the tissue. The cells were loosened from the tissue by blunt dissection and individual cells (approximately ten) were transferred with a microliter syringe to individual drops (100 μl) of HBS in a Falcon dish (Becton Dickenson Labware). The dish was situated on the inverted microscope stage (Nikon Diaphot with Hoffman modulation contrast optics and bright field illumination at 40X) and cells were selected for observation that met criteria established previously for viable cells (Zajic and Schacht, 1987). These included: (1) a cylindrical shape; (2) no swelling of the cell membrane; (3) a nucleus positioned at the base of the cell; (4) no Brownian motion of the organelles. Only cells meeting these criteria were used in the studies.

One cell in each drop was selected for continued observation and measurement. A VHS recorder connected to a video camera attached to the microscope recorded the entire experimental sequence. The drop was perfused with HBS at the rate of 100 μl/min utilizing a peristaltic pump and glass infusion and withdrawal pipettes placed in the drop. After 5 min of perfusion, test solutions (TSO: i.e., Hank's containing chemicals such as Ach) were introduced into the infusion tubing by placing it into TSO for 2 min, and then returning the tubing to HBS. The delay for delivery of the TSO to the drop was 5 min which allowed a total of approximately 10 min of observation and measurement of cell length before the TSO arrived at the cell and 2 min during the exposure to TSO. Recovery from any TSO effects to this first TSO exposure was monitored by maintaining the perfusion with HBS for an additional 10 min. In addition, all cells were tested at the end of the experiment with a second exposure to a TSO of trypan blue (0.2%, at min 20 to end) or to a 2 min exposure to a TSO of hypo-osmotic composition (min 22 to 24). If a cell did not exclude the dye or did not respond with a length change to the
hypo-osmotic TSO it was excluded from the study. All experiments were carried out at room temperature.

The drugs used as freshly prepared solutions with the pH adjusted when necessary were: acetylcholine chloride (Ach; Sigma), alpha-ketoglutaric acid (AKG; Sigma), eserine hemisulfate (physostigmine, Sigma), gamma-aminobutyric acid (GABA, Sigma), and carbamylcholine chloride (carbachol; Sigma). In the high potassium solutions (modified Hanks) NaCl was replaced by 50 and 100 mM KCl, respectively.

Computer hardware (Data Translation DT 2853) and software (DT-IRIS) was used to measure the cells (in pixel length calibrated with a stage micrometer) from single frames of the VHS at one min (min 1–10 and min 12–26) or 30 sec (min 10–12) time intervals.

Calibration was performed with a calibrated slide, measuring the distances on the slide with the computer from a video tape of the slide. The smallest incremental distance resolved by the measuring system was 0.3 μm. Graphs of the length of the cell over the time span of the experiment were constructed. Then three raters independently judged the graphs as to whether a cell length change occurred and in what direction it occurred. Where they did not agree a consensual agreement was made.

### Results

We studied only cells initially meeting the appearance criteria described by others (Zajic and Schacht, 1987). Some cells changed length during the first perfusion of the drop with HBS so that

### Table I

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Fig. 1. Length recordings from cells classified as unstable (see text for definition) and therefore excluded from further analysis. After 10 min of perfusion with a control HBS (min 0 to 10), various test HBS solutions (TSO) were perfused for 2 min (beginning at min 10 as indicated by the first arrow). Recovery was monitored by perfusing with control HBS for an additional 10 min (min 12–22). At the end of the experiment (min 22–24) a TSO of hypo-osmotic HBS (280 mOs) was perfused, as indicated by the second arrow, to test the cell's ability to contract. Recovery from the second TSO was occasionally monitored in response to perfusing with control HBS for an additional few min (min 24 to end).
Fig. 2. Cell length recordings illustrating the effects of perfusing test solutions (TSO) of different osmolarities and potassium concentrations. TSOs applied at 10–12 min were: iso-osmotic (a, 304 mOs HBS; b, 5 mM alpha-ketoglutarate; and c, 5 mM acetylcholine); hyperosmotic (d, 330 mOs HBS; e, 5 mM alpha-ketoglutarate-314 mOs; and f, 5 mM acetylcholine-314 mOs); hypo-osmotic (g, 280 mOs HBS); and 50 and 100 mM potassium (h, 50 mM K⁺; i, 100 mM K⁺). For additional explanation of the figure see legend for Fig. 1. No second TSO was tested at min 22 to 24 in d–i, instead trypan blue (0.2%) uptake was tested at min 21 and no length measurements were made.

In Fig. 2, different concentrations of test solutions were applied to the cells, along with initial and final solutions, as indicated. The recordings show changes in cell length over time.
additional criteria had to be developed. A change in length during the initial few min of HBS perfusion which we attributed to a stabilization process was acceptable. However, the cell had to achieve a constant length before application of the TSO. In addition, some cells changed length at a time when it could not be attributed to the TSO in the drop. Therefore, a criterion was added which stated that cells which underwent length changes that did not occur during the TSO or soon after were also classified as unstable. Fig. 1 illustrates recordings from cells which were judged as not meeting this “stability” criterion. One of the cells measured (Fig. 1, Exp 58, Cell 3) slowly shortened, then at min 19 the cell suddenly elongated. This is an example of a shape change which could not be attributed to the application of TSO. This particular cell extruded cytoplasm from just below the cuticular plate at min 19 and did not respond to the internal control of hypotonic TSO.

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**TABLE IV**

**THE NUMBER OF CELLS STUDIED AND THEIR RESPONSES TO THE APPLICATION OF TEST SOLUTIONS (TSO) OF 10 µM ACH WITH 10 µM ESERINE AND 10 AND 100 µM CARBACHOL APPLIED TO CELLS BEING BATHED BY A HBS OF THE SAME OSMOTIC PRESSURE (304 mOs)**

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<tr>
<td>TSO = 10 µM Carbachol</td>
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<td>0</td>
<td>9</td>
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<tr>
<td>TSO = 100 µM Carbachol</td>
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<td>0</td>
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In many cells, the start of the perfusion induced a shortening (Fig. 2a, 2d, 2e, 2f and 2h). After the cell had stabilized during the perfusion, addition of a TSO of HBS (304 mOs HBS at min 10 in Fig. 2a) did not change the cell’s length. A possible explanation is that the drops in the dish become hyperosmotic over time and the cells in the drop respond by slowly lengthening. When the perfusion is started the fresh HBS arriving at the drop returns the osmotic pressure of the drop to the original pressure and this, in turn, causes the cell to shorten to its original shape.

The application of hypo-osmotic solutions shortened the cells (Fig. 2g; Table I). Subsequently, the application of a hypo-osmotic solution was utilized as an internal control in several cells. For example (Fig. 2a), even though a cell...

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**TABLE V**

**THE NUMBER OF CELLS STUDIED AND THEIR RESPONSES TO THE APPLICATION OF TEST SOLUTIONS (TSO) OF 20 AND 100 µM GABA APPLIED TO CELLS BEING BATHED BY A HBS OF THE SAME OSMOTIC PRESSURE (304 mOs)**

<table>
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<td>TSO = 100 µM GABA</td>
<td>0</td>
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Fig. 4. Cell length recordings illustrating the effects of the perfusion of 10 μM carbachol. At 10–12 min iso-osmotic solutions of 10 μM carbachol were applied to cells being bathed by a control HBS of the same osmotic pressure (304 mOs). For additional explanation of the figure see legend for Fig. 1.
Fig. 5. Cell length recordings illustrating the effects of 100 μM carbachol. At 10–12 min iso-osmotic solutions of 100 μM carbachol were applied to cells being bathed by a control HBS of the same osmotic pressure (304 mOs). For additional explanation of the figure see legend for Fig. 1.
Fig. 6. Cell length recordings illustrating the effects of the 20 μM GABA. At 10–12 min iso-osmotic solutions of 20 μM GABA were applied to cells being bathed by a control HBS of the same osmotic pressure (304 mOs). For additional explanation of the figure see legend for Fig. 1.
Fig. 7. Cell length recordings illustrating the effects of 100 μM GABA. At 10–12 min iso-osmotic solutions of 100 μM GABA were applied to cells being bathed by a control HBS of the same osmotic pressure (304 mOs). For additional explanation of the figure see legend for Fig. 1.
does not respond to the application of fresh TSO solution (304 mOs HBS at min 10), the cell is still able to shorten to a subsequent application of hypo-osmotic solution (280 mOs HBS at min 22 in Fig. 2a). Increasing the osmotic pressure elongated the cells but at a lesser incidence than decreasing the osmotic pressure (Fig. 2d; Table I). Potassium chloride (50 mM, N = 7; 100 mM, N = 7) induced a reversible shortening of all OHCs tested (Fig. 2i and 2j; Fig. 3).

The addition of either 5 mM alpha-ketoglutarate (AKG) or Ach to HBS of either 290 mOs, 304 mOs or 314 mOs increased the osmotic pressure of the HBS by about 10 mOs. The OHCs elongated in response to 5 mM Ach (Fig. 3f, Table II) and the elongation was duplicated by 5 mM AKG (Fig. 3e, Table III). Experiments which applied 5 mM Ach or AKG iso-osmotic with the isolation and perfusion medium (HBS, 304 mOs) did not result in a detectable change in cell length (Fig. 3b and 3c; Tables II and III).

When Ach (10 µM) was applied together with eserine (10 µM), a cholinesterase inhibitor, it also induced no change in cell length (Table IV). We tested carbachol, an Ach agonist that is not hydrolyzed by cholinesterase. Carbachol at 10 and 100 µM did not change the length of the cells (Figs. 4 and 5, Table IV). In addition, GABA at 20 and 100 µM induced no change in the cell length (Figs. 6 and 7, Table V).

**Discussion**

Our results clearly indicate that neither Ach (5 mM, or 10 µM with eserine 10 µM), carbachol (10 or 100 µM), nor GABA (20 or 100 µM) induced a detectable change in the length of OHCs. This is in contrast to the results obtained with high potassium and changes in osmotic pressure which consistently altered OHC length in agreement with the previous results of others (for example, Zenner, 1986b; Dulan et al., 1988).

At present we can only speculate as to the reasons for the absence of a change in the length of the OHCs to the neurotransmitters of the efferent nerve fibers which synapse on the OHCs. Changing the membrane potential of OHCs alters their lengths in vitro: an increase in the membrane potential (hyperpolarization) increases their length, whereas a decrease (depolarization) decreases their length (Brownell, 1983, 1984; Brownell et al., 1985; Ashmore, 1987; Holley and Ashmore, 1988; Zenner, et al., 1985a, 1985b; Kachar et al., 1986; Santos-Sacchi and Dilger, 1988). The OHCs undergo a 0.02–0.03 µm maximal length change per mV change in the membrane potential (Ashmore 1987; Santos-Sacchi, 1989; Iwasa and Kachar, 1989) whereas a change in voltage near the resting membrane potential of the cell (−70 mV) results in less of a change in cell length (Santos-Sacchi, 1989). The maximum that the endocochlear potential responds to efferent stimulation is about 5 mV (Fex, 1966; Konishi and Slepian, 1971). This may be used cautiously as an estimate of the possible change in the hair cell potential. Under this assumption, an OHC membrane potential change of 5 mV would result in a hair cell length change of 0.1-0.15 µm. This change is an order of magnitude less than that reported by others for the shortening due to Ach (e.g., > 1 µm by Slepecky et al., 1988b). Also, it is less than the resolution of our present system (0.3 µm). Thus, our negative results with length measurements are consistent with the electrophysiological data.

Several laboratories have reported that potassium shortens isolated outer hair cells (Goldstein and Mizukoshi, 1967; Brownell et al., 1985; Slepecky et al., 1988b, Zenner et al., 1988; Slepecky et al., 1988a; Dulan, et al., 1988) with no negative reports appearing in the literature. Hypo-osmotic solutions likewise shorten isolated OHCs (Dulan, et al., 1988). While some laboratories (Dulan et al., 1987; Zenner et al., 1985b) observed that OHCs elongate in response to hyperosmotic solutions, other reports (Slepecky et al., 1988a) indicate that OHCs do not change length, though they shrink and wrinkle, in response to hyperosmotic solutions. We studied potassium and osmotic pressure to define our preparation and to reconcile the differences others have reported.

Our results confirmed that 50 and 100 mM KCl induced a reversible shortening of the isolated OHCs, since the effect was readily reversed by washing away the TSO of KCl with HBS after 2 min of application. We also observed a shortening of the cells in response to hypo-osmotic solutions consistent with the results of Dulan et al. (1987, 1988). The hyperosmotic solutions did induce the
wringling reported by others and elongate 50% of the cells studied. This confirms the results of Zenner et al. (1985b) who found that the length change to hyperosmotic solutions was less consistent than the shortening to hypo-osmotic solutions, but is inconsistent with the lack of change in length found by others (Slepecky et al., 1988a).

There may be several reasons why our results differ from previous reports. One of the major problems with isolated HC work is that most investigators apply anatomical or cell appearance criteria only in selecting cells for their experiments. One of the major differences between our preparation and that of others is that we introduced the additional criterion of stability during the perfusion. Many of our cells changed length before or much later than the time when the TSO arrived at the drop. Such cells were placed in the ‘unstable’ category and so excluded from the study. Several cells in this category initiated a ‘shortening’ at the beginning of the experiment and continued to slowly shorten over time. Some of these even returned to the original length and others returned beyond the original length. If we had applied drug at the beginning of the experiment we would have attributed many of these length changes to the action of the drug with even the occurrence of recovery. This might, in part, account for the ‘positive’ results of others and our ‘negative’ results. Brownell (Brownell, 1983, 1984; Brownell et al., 1985) applied Ach by iontophoresis to isolated OHCs. He did not quantify the length change except to say it was smaller than the changes they observed in response to applied voltage gradients. According to Slepecky et al. (1988b) 5 mM Ach applied to a drop containing the isolated OHCs shortens the cells. Neither study substantiated the observed effect as cholinergic since they did not provide pharmacological controls such as testing cholinergic agonists with much less potency (e.g., choline) or testing cholinergic antagonists against the shortening. We observed an increase in the length of our cells when duplicating their experimental design. Slepecky et al. (1988b) did not adjust the osmotic pressure of the 5 mM Ach solution. We tested Ach 5 mM after adding the drug to 290 mOs HBS (Slepecky et al., 1988b) as well as adding the drug to 304 mOs HBS (the basic osmolarity in our study). Under both conditions the solutions of Ach increased the length of the OHCs. We surmise that the elongation is caused by the solutions of Ach being hyperosmotic (by 10 mOs) with respect to the HBS bathing the cell when the TSO is applied. Supporting this conclusion is the observation that the elongation was duplicated by 5 mM AKG, a molecule which has no activity at any receptor and increased the osmotic pressure of the solution. Finally, the length change was not observed in response to TSOs of 5 mM Ach or 5 mM AKG iso-osmotic with the HBS bathing the cells before their application. Therefore, any length changes observed under conditions of uncontrolled osmotic pressure should be considered artifacts and not cholinergic phenomena.

Ach is readily hydrolysed by cholinesterase and the absence of an effect may thus be due to its rapid breakdown. It is not known whether the enzyme is active or present in isolated OHCs. To inhibit the enzyme we tested Ach with eserine added to the solution. In these cases the cells still did not change their length. Another alternative is to test carbachol which is not hydrolysed by the esterase and yet is active at the “nicotinic-like” receptors utilized by the efferents in the guinea pig cochlea (Bobbin and Konishi, 1974). Ten and 100 μM carbachol induced no detectable change in the length of the cells. These concentrations are close to those effective in the cochlea (50 μM; Bobbin and Konishi, 1974) and in the lateral line (10 μM; Bobbin et al., 1985) and so should have been effective in the isolated cells. Thus neither Ach nor the Ach agonist, carbachol, induced changes in the length of the OHCs isolated. Therefore, our results with both agents are consistent and suggest alternate interpretations for the changes found by others (Brownell et al., 1985; Slepecky et al., 1988b; Plinkert and Zenner, 1989).

Of additional interest are the effects of efferent neurotransmitter substances other than Ach on the length of isolated OHCs. Although Ach is the transmitter of the majority of the efferent neurons innervating the cochlea, a subpopulation of efferents may utilize GABA as a transmitter (see reviews by: Altschuler and Fex, 1986; Bledsoe et al., 1988). GABA-containing efferent nerve fibers are localized mostly in a small population of efferent neurons innervating the OHCs of the third and
first half of the fourth turns in the mammalian cochlea (Fex and Altschuler, 1984; Altschuler and Fex, 1986; Fex et al., 1986; Eybalin, et al., 1988). Also, some OHCs appear to be innervated by both GABA and Ach containing efferents (Altschuler and Fex, 1986). Although GABA is a major neurotransmitter in the vertebrate central nervous system, its role in synaptic processes of octavolateralis sensory organs is unresolved. Affecting OHC motility could be a role for GABA. However, we could not detect a change in the length of isolated OHCs to the application of GABA.

To date there are no reports of in vivo recordings from OHCs and their responses to efferent stimulation. Therefore, we do not know what properties of the OHCs are changed by the efferents. One possibility is that the efferent neurotransmitter(s) changes a cell constituent (e.g., cyclic AMP, inositol triphosphate) without shortening or elongation of the hair cell. On the other hand, indirect evidence suggests that the efferent neurotransmitter interacts with receptors on OHCs, altering an ion channel and so induce a depolarization or hyperpolarization of the cell. For cochlear hair cells, Desmedt and Robertson (1975) suggest that the efferent neurotransmitter opens a chloride channel to allow chloride to enter the cell. For turtle hair cells (Art et al., 1982) and toadfish saccule hair cells (Steinacker and Rojas, 1988) it appears that the neurotransmitter opens a potassium channel and so allows potassium to leave the cell. Both ion fluxes would hyperpolarize the cell and presumably result in cell elongation. Flock and Russell (1973) recorded a hyperpolarization of the sensory hair cell of fish lateral line organ. On the other hand, it is possible that the efferent neurotransmitter acts on an ion channel which causes depolarization (e.g., calcium influx), resulting in cell shortening. In fact, Housley et al. (1990) find both depolarization and hyperpolarization responses to efferent transmitter application in the frog semicircular canal hair cells. In any case, as discussed earlier, if the efferent neurotransmitter depolarizes or hyperpolarizes the OHCs, the cell length would change, but the change in length would presumably be less than measured to date.

In summary, the literature has described many agents and treatments that alter the lengths of isolated OHCs by over 1 μm. Among them are changes in osmotic pressure, potassium concentration, and the efferent neurotransmitter, Ach. In our study, the cells did respond as expected to changes in osmotic pressure and potassium concentration. However, we were unable to detect a change in the lengths of the cells that could be attributed to the pharmacological actions of Ach, carbachol or GABA. We conclude that if Ach, carbachol or GABA did induce a length change it was less than 0.3 μm, the resolution of our methods.

Acknowledgements

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References


